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Ca^{2+}/Mg^{2+}-dependent endonuclease activation is an early event in VP-16-induced apoptosis of human breast cancer MCF7 cells in vitro

Irina A. Sokolova, Kenneth H. Cowan, Erasmus Schneider *

Medicine Branch, National Cancer Institute, Bethesda, MD 20892, USA

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Abstract

Apoptosis is now recognized as one of the major processes regulating the size of cell populations. However, despite intensive investigations the biochemical and enzymological mechanisms involved in apoptosis remain unclear. In the present study we demonstrate activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease during VP-16-induced apoptosis in MCF7 cells. Nuclease activation occurred prior to the appearance of internucleosomal DNA fragmentation, suggesting that this activation may be an early and possibly critical step in drug-induced apoptosis. Analysis of the internucleosomal DNA fragments showed that they contained phosphorylated 5'-ends, indicating that they were produced by a Ca^{2+}/Mg^{2+}-dependent endonuclease.

Keywords: Endonuclease; Apoptosis; DNA fragmentation

1. Introduction

Apoptosis was first described by Kerr and co-workers as a form of cell death that is characterized by a series of morphological changes and internucleosomal DNA degradation (DNA laddering) [1,2]. Since internucleosomal DNA degradation was generally considered a hallmark of apoptosis, it was assumed that a nuclear endonuclease had to be activated for apoptosis to occur [2–4]. Of the many different endonuclease activities identified, a Ca^{2+}/Mg^{2+}-dependent endonuclease was considered the most likely candidate for the 'apoptotic' nuclease, since several studies have found that internucleosomal DNA fragmentation of chromatin occurred during in vitro incubation of isolated cell nuclei in the presence of both Ca^{2+} and Mg^{2+} ions [4–7]. Evidence that a similar nuclease activity may also be responsible for internucleosomal DNA degradation in vivo came from studies which suggested that increased intracellular calcium levels were sufficient to activate DNA degradation during apoptosis [8–11]. Subsequent studies have identified an endonuclease activity(ies) that was Ca^{2+}- and/or Mg^{2+}-dependent and was able to cleave chromatin into oligonucleosomal DNA fragments in vitro [12–15]. However, alternate nucleases have also been proposed as candidates for DNA degradation during apoptosis [16–19].

Recent advances in the understanding of the molecular mechanisms of apoptosis suggested that the typical DNA degradation into oligonucleosomal fragments (DNA laddering) may only be the endproduct of a cascade of stepwise DNA degradations. There is now evidence to suggest that chromatin degradation during apoptosis occurs in distinct steps: an initial cleavage into high molecular weight fragments of approx. 300 kbp, followed by a further degradation into approx. 50 kbp fragments and finally into the typical oligonucleosomal ladder [20–23]. Whether these distinct cleavage steps are mediated by one or several nucleases is not known. Furthermore, whether nuclease activation must precede the onset of apoptosis, or whether it is the result of the induction of apoptosis is unclear.

Many, if not all, tissues rely on the balance between cell proliferation and cell death to maintain homeostasis. The loss of this balance is thought to be responsible for neoplastic growth. While traditionally studies of tumor biology have concentrated on an increased rate of cell prolifer-

Abbreviations: VP-16, etoposide; 5-FdUrd, 5-fluoro-2'-deoxyuridine; DAPI, 4',6-diamino-2-phenylindole; CIAP, calf intestinal alkaline phosphatase; SDS, sodium dodecyl sulfate; PBS, phosphate buffer solution; TE buffer, 10 mM Tris-HCl/1 mM EDTA (pH 8.0); TAE buffer, 40 mM Tris-acetate/2 mM EDTA (pH 7.8).

* Corresponding author. E-mail: eschneid@pop.nih.gov. Fax: +1 (301) 4020172.

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ation as the cause for the increase in tissue mass, more recently the concept was proposed in which a reduction in the rate of natural cell death leads to a subsequent increase in tumor mass. Furthermore, studies have demonstrated that many anticancer drugs such as etoposide, methotrexate, cisplatin, chlorambucil and 5-FdUrd can induce apoptosis in tumor cells in vivo and in vitro [24–26].

Despite intensive research, the exact biochemical pathways to and the molecules involved in apoptosis remain elusive. The widespread appearance of internucleosomal DNA degradation during apoptosis suggests that an endonuclease may be critically involved in this process. In the present study we present evidence indicating that activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease was involved in VP-16-induced apoptosis in human mammary carcinoma MCF7 cells and that this activation preceded the appearance of internucleosomal DNA degradation. Results from this study have been presented in abstract form at the 94th annual meeting of the American Association for Cancer Research, 1994.

2. Materials and methods

2.1. Materials

DMEM medium, antibiotics solution, fetal bovine serum, pBR322, calf intestinal alkaline phosphatase (CIAP) and T4 polynucleotide kinase were purchased from Gibco/BRL (Gaithersburg, MD). [γ-^{32}P]ATP was purchased from New England Nuclear (Wilmington, DE), and RNase A and proteinase K were obtained from Boehringer-Mannheim (Indianapolis, IN). DAPI was obtained from Sigma (St. Louis, MO). VP-16 was kindly donated by Dr. B.K. Sinha, National Cancer Institute (Bethesda, MD).

2.2. Cell culture

MCF7 human breast cancer cells were maintained at 37° C in 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 U/ml).

2.3. VP-16 treatment

MCF7 cells were plated into 135-mm culture dishes at a concentration of 10^6 cells per dish and maintained for 3–4 days, after which time they were in logarithmic growth phase. At the beginning of the experiment (day 0) the medium was replaced and 50 μM VP-16 (experimental cells) or 0.05% DMSO (control cells) were added. Day 0 cells were harvested directly after medium replacement. During the next 8 days experimental (drug-treated) and control (DMSO only-treated) cells were collected daily from the culture supernatant by centrifugation and combined with cells that had remained attached to the culture dish and were harvested by scraping. The combined cells were washed in PBS and counted with a Coulter Counter. Cells were stored as a pellet at −20° C until further analysis.

2.4. Isolation of cell nuclei

Isolation of cell nuclei was performed essentially as previously described [15]. Briefly, 10^7 cells were suspended in 800 μl ice-cold buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, 0.1% Triton X-100, 0.9 M sucrose and homogenized with 5–7 strokes of a motorized glass/teflon homogenizer. The homogenate was overlaid onto a sucrose cushion containing 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, 1.2 M sucrose at a ratio of 2 vols. of homogenate to 1 vol. of sucrose cushion. Samples were centrifuged for 30 min at 750 × g at 4° C in a swinging-bucket rotor. The pellet, containing the cell nuclei, was resuspended in 200 μl buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, 0.25 M sucrose, 50% glycerol and stored at −20° C.

2.5. Nuclear extract

To prepare nuclear extracts an aliquot of cell nuclei was pelleted and resuspended in 10 mM Tris-HCl (pH 8.0) at a DNA concentration of 5 mg/ml. Three volumes of extraction buffer (10 mM Tris-HCl (pH 8.0), 0.4 M KCl, 0.1% Triton X-100) were then added to the suspension of cell nuclei and nuclear proteins were extracted for 60 min at +4° C, followed by centrifugation at 24000 × g for 30 min. The supernatant was used as nuclear extract and stored at −20° C.

2.6. Detection of endonuclease activity

To detect endonuclease activity, 0.5 μg of supercoiled plasmid pBR322 DNA were digested with nuclear extract containing 0.5 μg or 1 μg of protein in 30 μl incubation buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) for 30 min at 37° C. These concentrations of $MgCl_2$ and $CaCl_2$ had been previously found to give maximal endonuclease activity [15]. After 30 min proteinase K and SDS were added to a concentration of 0.1 mg/ml and 1%, respectively, and incubation was continued for 30 min at 37° C. The DNA was then extracted with phenol/chloroform/isoamyl alcohol (50:49:1) and analyzed by electrophoresis on a 1% agarose gel containing 2 μg/ml of ethidium bromide in Tris-acetate-EDTA buffer (TAE). The gel was photographed under UV-light using Polaroid type 57 (positive) or 55 (negative) film. Negatives were scanned on a densitometer and nuclease activity was quantitated as the amount of linear DNA produced.

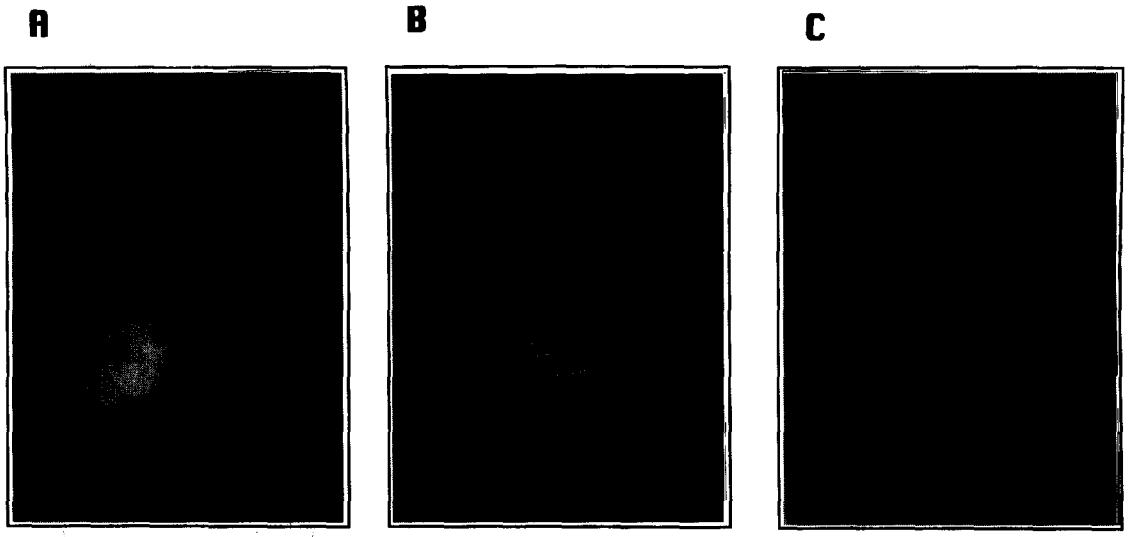

Fig. 1. Fluorescence microscopy of DAPI stained MCF7 cells before (A) and after (B and C) 8 days of VP-16 treatment. Cells were stained with DAPI as described in Section 2.

2.7. DNA laddering analysis

$2 \cdot 10^6$ cells were lysed in 500 μl of lysis buffer (5 mM Tris-HCl (pH 7.4), 20 mM EDTA, 0.5% Triton X-100) on ice for 2 h, followed by centrifugation at 30 000 $\times$ g for 30 min. The supernatants were treated with RNase A (0.5 mg/ml) for 60 min at 50° C, followed by proteinase K (0.4 mg/ml) in the presence of 1% SDS for another 60 min at 50° C, extracted twice with phenol/chloroform/isoamyl alcohol, and precipitated with ethanol. The DNA was dissolved in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE). 2 μg of DNA were loaded onto a 1.8% agarose gel and electrophoresed in TAE buffer for 2 h at 100 V.

2.8. Analysis of DNA ends

To dephosphorylate the 5'-ends of cleaved DNA, 5 μg DNA was incubated in calf intestinal alkaline phosphatase buffer in the presence of 5 units of CIAP at 37° C for 1 h. The reaction was stopped by heating to 75° C for 10 min

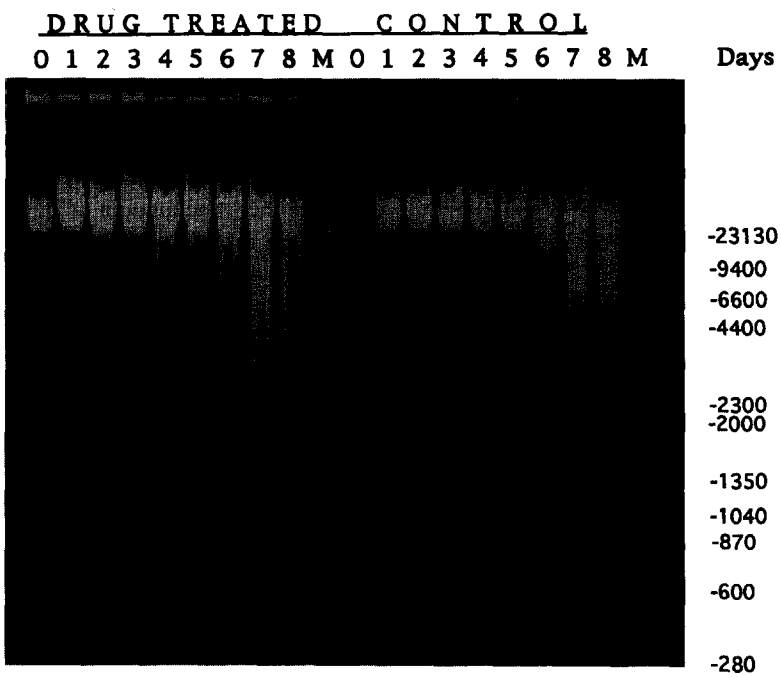

Fig. 2. Analysis of DNA fragmentation in MCF7 cells treated with 50 μM VP-16 for 8 days. Cells were harvested daily and DNA was isolated as described in Section 2 and separated on a 1.8% agarose gel. M: λ/*Hind* III DNA fragments as molecular weight markers.

and the DNA was purified by extraction with phenol/chloroform/isoamyl alcohol and precipitation with ethanol. 1 μg of this DNA was then end-labeled by incubation in 20 μl reaction buffer (60 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.33 μM ATP, 15 mM 2-mercaptoethanol) with 5 units of T4 polynucleotide kinase in the presence of 100 μCi of [γ-^{32}P]ATP at 37° C for 2 h. The reaction was stopped by adding 1 μl of 0.5 M EDTA. The labeled DNA was separated from unincorporated nucleotides by filtration on Miniprep Spun Columns (Pharmacia P-L Biochemicals) in accordance with the manufacturer's instructions. 0.2–0.3 μg of the end-labeled DNA were then used for analysis by agarose gel electrophoresis, followed by autoradiography.

2.9. DAPI staining of MCF 7 cells

Approx. 10^6 cells were trypsinized, washed once in PBS and fixed in 70% ethanol. Fixed cells were centrifuged, resuspended in 2 ml pepsin (0.5%) and incubated for 10 min at room temperature. Then 2 ml DAPI (5 μg/ml) and 0.2 ml RNase (1 mg/ml) were added and incubated for 20 min. Cells were examined by fluorescence microscopy.

2.10. Protein measurements

Protein concentrations were measured by the method of Bradford [27].

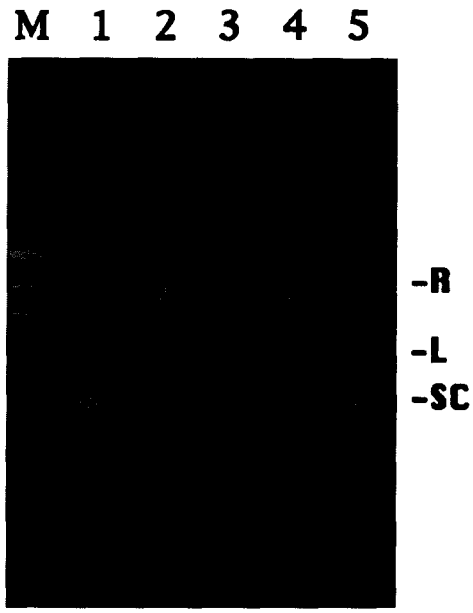

Fig. 3. Characterization of the endonuclease activity in nuclear extracts from MCF7 cells. Nuclear extract (1 μg of protein) from MCF7 cells treated with VP-16 for 8 days was incubated with 0.5 μg of plasmid pBR322 DNA in reaction buffer containing 10 mM Tris-HCl (pH 8.0) and different divalent cations: lane 1: plasmid DNA only incubated in buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$, but no nuclear extract; lane 2: 10 mM $MgCl_2$; lane 3: 1 mM $CaCl_2$; lane 4: 10 mM $MgCl_2$ andl mM $CaCl_2$; lane 5: the same as in lane 4, but also with 5 mM EDTA and 5 mM EGTA. After incubation for 30 min, the samples were treated as described in Section 2, and the DNA was separated on a 1% agarose gel in TAE buffer in the presence of 2 μg/ml ethidium bromide. SC, L, R: supercoiled, linear and relaxed forms of plasmid DNA, respectively.

3. Results

3.1. Effect of VP-16 on MCF7 cells

DAPI staining and fluorescence microscopy were used to assess whether drug-induced cytotoxicity in MCF7 cells was accompanied by morphological changes associated with apoptosis. As shown in Fig. 1, untreated MCF7 cells displayed uniformly stained nuclei (Fig. 1A). In contrast, following 8 days exposure to VP-16, MCF7 cells exhibited the classical morphological features of apoptosis such as chromatin margination at the nuclear periphery (Fig. 1B) and blebbing of the cell nucleus (Fig. 1C).

3.2. DNA fragmentation in MCF7 cells after VP-16 treatment

Since MCF7 cells that had been exposed to VP-16 displayed morphological features typically associated with apoptosis, we next examined the effect of VP-16 treatment on internucleosomal DNA fragmentation. DNA laddering became apparent after 4 days of drug treatment and gradually increased during further drug exposure (Fig. 2). In contrast, DNA laddering was not observed in untreated control cells until day 7, when some DNA degradation into longer oligonucleosome fragments was observed. This may represent some cell death due to nutrient depletion during the 7 days in culture. A semiquantitative analysis of DNA fragmentation after 8 days revealed that approx. 6-times more control cells than VP-16-treated cells were required to give the same amount of fragmented DNA. Thus, VP-16 exposure resulted in significantly greater DNA fragmentation that occurred earlier than in control cells.

3.3. Endonuclease activity in MCF7 cells

Preliminary experiments revealed the presence of endonuclease activity in nuclear extracts from VP-16-treated MCF7 cells. In order to characterize this activity, we examined the effect of different divalent cations on nuclease activity. As shown in Fig. 3, nuclease activity in nuclear extracts from VP-16-treated MCF7 cells was readily detected in the presence of 10 mM $MgCl_2$ (lane 2). Incubation with 1 mM $CaCl_2$ (lane 3) also produced some nuclease activity, but maximal activity was observed in the presence of both cations (lane 4). In contrast, incubation in the presence of EGTA and EDTA abolished this activity

(lane 5). This indicated that the nuclease activity in MCF7 cells was Ca^{2+}/Mg^{2+}-dependent.

In order to determine whether drug-induced apoptosis and DNA laddering were associated with increased endonuclease activity, nuclear extracts from MCF7 cells treated with VP-16 for different lengths of time were assayed for endonuclease activity. As shown in Fig. 4B, low levels of nuclease activity were present in control untreated MCF7 cells (compare lanes C with lanes 0). However, when MCF7 cells were treated with VP-16, an approx. 3-fold increase in endonuclease activity was observed after 2 days of drug treatment (Fig. 4A), when more than 90% of the cells still excluded Trypan blue and remained attached to the culture dish. This activity continued to increase up to approx. 10-fold over control levels after 8 days of VP-16 treatment (Fig. 4C). In contrast, no increase of nuclease activity over basal levels was found in MCF7 cells that were maintained in the absence of VP-16. Thus, the drug-induced apoptosis in MCF7 cells was accompanied by an increase in endonuclease activity which preceded the appearance of internucleosomal DNA fragmentation (compare Fig. 2 with Fig. 4).

3.4. Characterization of the endonuclease cleavage products

The products of endonuclease cleavage are characteristic for the type of endonuclease catalyzing the reaction. Ca^{2+}/Mg^{2+}-dependent endonuclease produces DNA fragments with 5′-P and 3′-OH ends, whereas acid endonucleases produce DNA fragments with 5′-OH and 3′-P ends [28]. To confirm that the VP-16-induced DNA fragmentation in MCF7 cells was indeed mediated by a Ca^{2+}/Mg^{2+}-dependent endonuclease and not by an acid endonuclease, as suggested by some studies [17,29], the 5′-ends of the DNA fragments formed in MCF7 cells

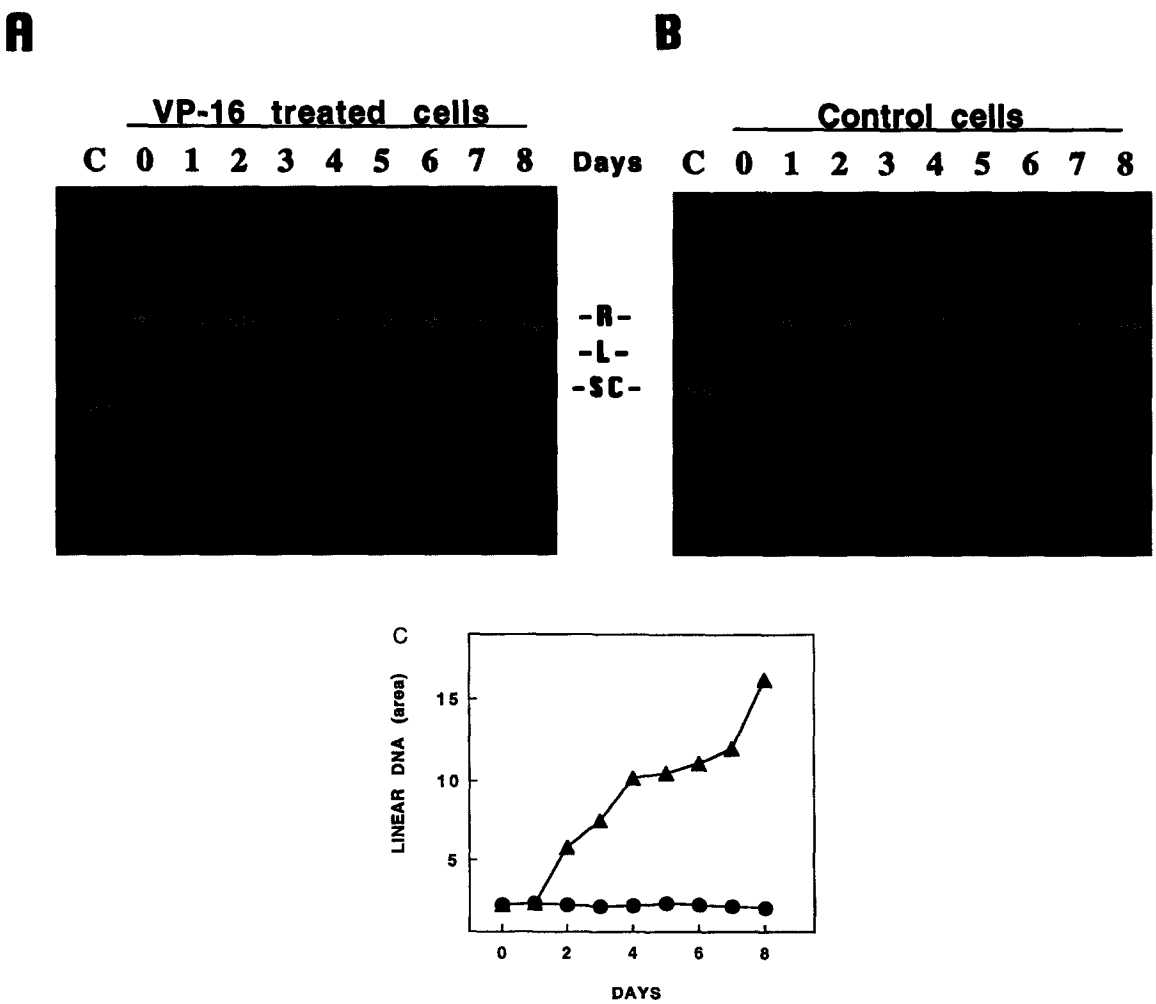

Fig. 4. Endonuclease activity in nuclear extracts from MCF7 cells treated with (A) or without (B) 50 μM VP-16. Nuclear extracts (1 μg of protein) were incubated with 0.5 μg of plasmid pBR322 DNA in incubation buffer (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM $CaCl_2$) for 30 min. After incubation, the samples were treated as described in Section 2 and the DNA was separated on a 1% agarose gel in TAE buffer in the presence of 2 μg/ml ethidium bromide. SC, L, R: supercoiled, linear and relaxed forms of plasmid DNA, respectively. (C) Densitometric quantitation of the amount of linear DNA in the gels shown in (A) and (B); (●) nuclear extract from control cells, (▲) nuclear extract from experimental cells. The gels shown were photographed and the negatives scanned on a densitometer. Nuclease activity was measured as the amount of linear DNA.

during VP-16 treatment were examined. DNA isolated from MCF7 cells at day 0 and after 8 days with or without VP-16 was treated with T4 kinase in vitro in the presence of [γ-^{32}P]ATP. As shown in Fig. 5B, only the DNA isolated from cells after 8 days in culture became labeled. Furthermore, labeling was only detected after dephosphorylation of the 5'-ends with alkaline phosphatase, indicating that the 5'-ends were originally phosphorylated. This result was consistent with the assumption that intracellular DNA degradation during apoptosis was due to a Ca^{2+}/Mg^{2+}-dependent endonuclease. Although some labeling was also detected in DNA isolated from untreated cells after 8 days, at least 5-fold larger amounts of DNA had to be loaded onto the gel in order for the labeling to become detectable (Fig. 5) and only fragments with higher molecular weight were labeled. In addition, phosphatase-dependent 5'-end labeling of higher molecular weight fragments from VP-16-treated cells was detected as early as day 2, when enzymatic activity started to increase (data not shown). These results further confirmed that VP-16 treatment of MCF7 cells induced DNA fragmentation much more extensive than occurred through nutrient depletion alone.

To determine that the Ca^{2+}/Mg^{2+}-dependent endonuclease detected in nuclear extracts from MCF7 cells by our in vitro assay was of the same type as the one that produced DNA fragmentation in whole cells, we digested plasmid pBR322 DNA in vitro with extracts of cell nuclei prepared after 2 and 8 days of VP-16 treatment under two different reaction conditions: (1) neutral condition (10 mM Tris-HCl, pH 8.0) in the presence of 10 mM $MgCl_2$ and 1 mM $CaCl_2$ (our standard condition) or (2) acid condition (50 mM Na-acetate, pH 5.4) without divalent cations. The digested plasmid DNA was then treated with T4 kinase with or without prior alkaline phosphatase treatment. The results of this experiment are shown in Fig. 6. Under the condition favoring Ca^{2+}/Mg^{2+}-dependent endonuclease activity, the cleaved plasmid DNA was labeled only after prior alkaline phosphatase treatment, confirming that the enzyme present in nuclear extracts cleaved DNA with 5'-P and 3'-OH ends. In contrast, DNA digested under the acid condition was equally labeled with and without prior alkaline phosphatase treatment. Therefore, we concluded that the endonuclease responsible for DNA degradation in MCF7 cells is a Ca^{2+}/Mg^{2+}-dependent endonuclease.

4. Discussion

It is now widely assumed that apoptosis is responsible for anticancer drug-induced cell death. However, relatively little is known about the exact mechanisms that lead to drug-induced apoptosis. The prominence of internucleosomal DNA degradation has led to the hypothesis that a nuclear endonuclease was required for apoptosis to occur and that its activation was an early and essential step and required de novo protein synthesis. The enzyme most commonly regarded as the apoptotic endonuclease is a

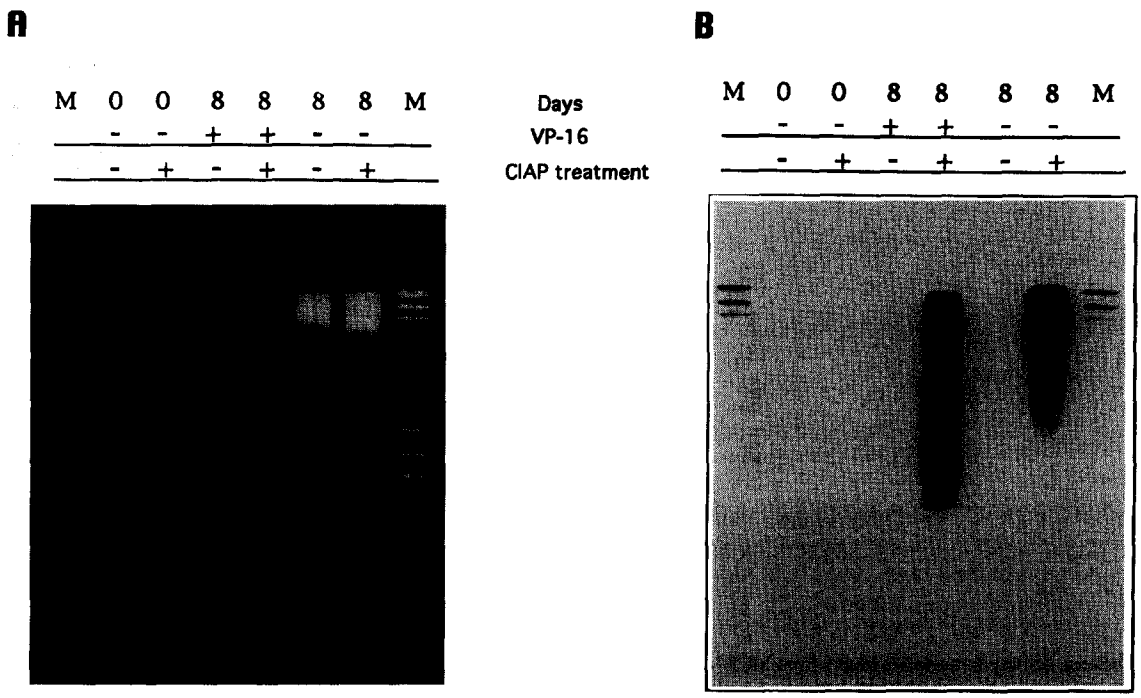

Fig. 5. 5' end labeling of cellular DNA cleaved in vivo. (A) cellular DNA was isolated as described in Section 2 for DNA laddering analysis, then 5'-end labeled with T4 kinase with or without prior alkaline phosphatase treatment. 0.1 μg and 0.5 μg labeled DNA from VP-16-treated and control cells, respectively, was separated on a 1.8% agarose gel in TAE buffer, stained with ethidium bromide and photographed. (B) autoradiogram of the gel shown in A. M: end-labeled λ/*Hind* III DNA fragments.

Ca^{2+}/Mg^{2+}-dependent endonuclease [12,13,30–32], although a number of other enzymes has recently also been suggested to play a role in apoptosis [16–18,33].

Few studies have directly compared DNA degradation and endonuclease activity. Activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease during apoptosis was shown in rat prostate cells in vivo after castration [9] and in nutrient-depleted murine myeloma cells [32]. In the present study we demonstrate that a Ca^{2+}/Mg^{2+}-dependent endonuclease activity increased markedly after VP-16 treatment of human mammary tumor MCF7 cells in vitro. Using specific end-labeling of the cleavage products, we have further confirmed that this endonuclease activity was a Ca^{2+}/Mg^{2+}-dependent endonuclease. In addition, the increase in nuclease activity preceded the appearance of internucleosomal DNA fragmentation by 2–3 days. Thus, one could speculate that cytotoxic drug treatment stimulated a Ca^{2+}/Mg^{2+}-dependent endonuclease activity, either directly or indirectly, which then in turn promotes and/or accelerates cell death via apoptosis. The mechanism(s) of endonuclease activation remain unclear. Isaacs and co-workers have proposed that activation occurs via a rapid calcium ion influx into the injured cells [9]. However, other mechanisms such as changes in phosphorylation of the endonuclease or some putative regulatory protein have been suggested by studies showing that apoptosis could be prevented by inhibitors of phosphatases [34,35].

It has recently been suggested that an early and possibly critical event in apoptosis was the cleavage of chromatin into 300 kbp fragments followed by further cleavage into 50 kbp fragments [21,23]. The enzyme involved in this initial fragmentation has not been identified. Our observation that the increase in Ca^{2+}/Mg^{2+}-dependent endonuclease activity preceded the appearance of the small oligonucleosomal fragments in VP-16-treated MCF7 cells suggested a possible role for the Ca^{2+}/Mg^{2+}-dependent endonuclease in the early steps of drug-induced apoptosis. However, Brown et al. [22] recently suggested that the

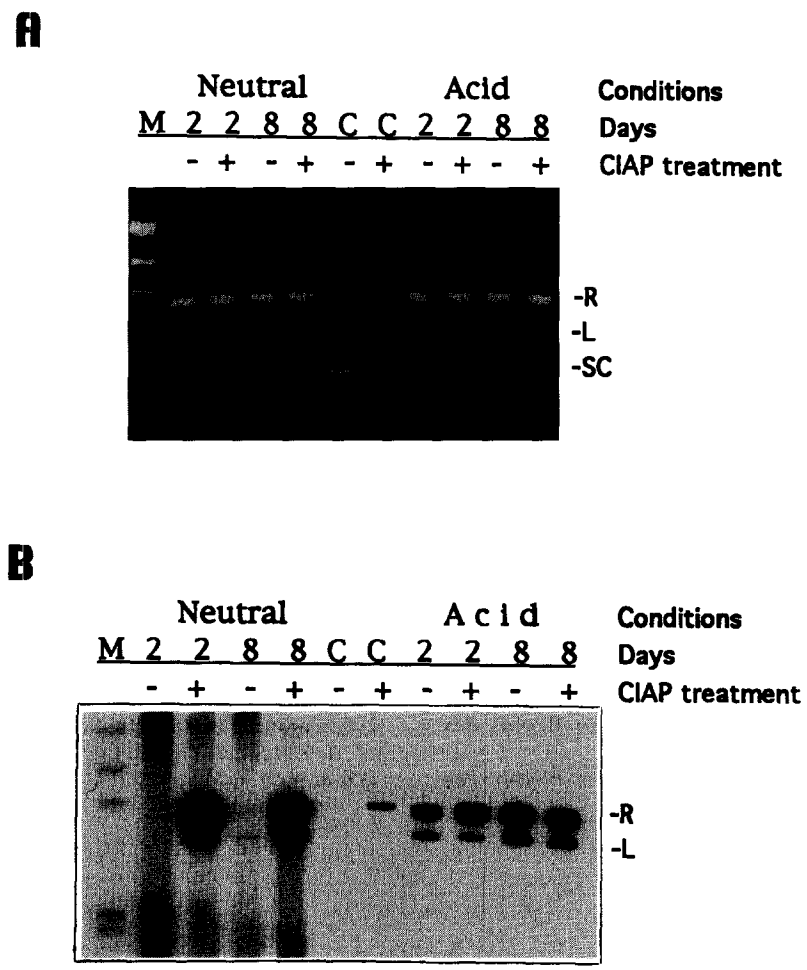

Fig. 6. 5′ end labeling of plasmid pBR322 DNA cleaved in vitro by nuclear extract from VP-16-treated cells under neutral or acid conditions. (A) 0.5 μg of plasmid pBR322 DNA was incubated with 1 μg of nuclear extract protein from MCF7 cells treated with 50 μM VP-16 for 2 or 8 days under two different conditions: (1) neutral condition, containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$, or (2) acid condition, containing 50 mM Na-acetate (pH 5.4). After incubation the samples were treated with proteinase K, extracted with phenol/chloroform/isoamyl alcohol and the DNA was precipitated with ethanol. The DNA of each sample was 5′-end labeled with or without prior alkaline phosphatase treatment and then separated on a 1% agarose gel in TAE buffer. (B) autoradiogram of the gel shown in (A). M: end-labeled λ/*Hin*d III DNA fragments.

enzyme involved in the earliest stages of high molecular weight DNA degradation had characteristics that differed from those of the Ca^{2+}/Mg^{2+}-dependent endonuclease. Further progress on this question awaits the identification and isolation of the nuclease(s) involved.

Our study furthermore demonstrated that human breast cancer MCF7 cells could undergo apoptosis following treatment with VP-16 in vitro. MCF7 cells stained with the DNA chromophor DAPI after 8 days of VP-16 treatment showed the classical morphological features of apoptosis, i.e., margination of chromatin at the nuclear periphery, chromatin condensation and fragmentation of the cell nucleus. In addition we also found clear internucleosomal DNA fragmentation (DNA laddering) which is widely regarded as a hallmark of apoptosis. In contrast, a recent study by Oberhammer et al. [23] failed to detect any evidence for apoptosis in MCF7 cells that were serum-depleted for 2 h. While it is difficult to compare the two results because of the different experimental conditions used, it is conceivable that the mechanism(s) of cell death induced by VP-16 is different from that induced by starvation. Further experiments are needed to resolve this discrepancy.

Taken together, our experiments indicated that the enzyme activity induced by VP-16 treatment and presumably responsible for the internucleosomal DNA fragmentation is a neutral Ca^{2+}/Mg^{2+}-dependent enzyme. We have furthermore shown that stimulation of a Ca^{2+}/Mg^{2+}-dependent endonuclease activity preceded the appearance of small oligonucleosomal DNA fragments, suggesting that activation of this enzyme may be an early and possibly critical step in drug-induced apoptosis. In addition, we have demonstrated that VP-16 treatment of human mammary MCF7 cells in culture led to cell death by apoptosis.

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